ASN NEURO RESEARCH ARTICLE

Supplementary data

ASN NEURO 2(1):art:e00028.doi:10.1042/AN20090055

Olfactory discrimination training up-regulates and reorganizes expression of microRNAs in adult mouse hippocampus

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Cite this article as: Smalheiser NR, Lugli G, Lenon AL, Davis JM, Torvik VI and Larson J (2010) Olfactory discrimination training up-regulates and reorganizes expression of microRNAs in adult mouse hippocampus. ASN NEURO 2(1):art:e00028.doi:10.1042/AN20090055

ABSTRACT

Adult male mice (strain C57Bl/6J) were trained to execute nose-poke responses for water reinforcement; then they were randomly assigned to either of two groups: olfactory discrimination training (exposed to two odours with reward contingent upon correctly responding to one odour) or pseudo-training (exposed to two odours with reward not contingent upon response). These were run in yoked fashion and killed when the discrimination-trained mouse reached a learning criterion of 70% correct responses in 20 trials, occurring after three sessions (a total of ~40 min of training). The hippocampus was dissected bilaterally from each mouse ($N=7$ in each group) and profiling of 585 miRNAs (microRNAs) was carried out using multiplex RT–PCR (reverse transcription–PCR) plates. A significant global up-regulation of miRNA expression was observed in the discrimination training versus pseudo-training comparison; when tested individually, 29 miRNAs achieved significance at $P=0.05$. miR-10a showed a 2.7-fold increase with training, and is predicted to target several learning-related mRNAs including BDNF (brain-derived neurotrophic factor), CAMK2b (calcium/calmodulin-dependent protein kinase IIβ), CREB1 (cAMP-response-element-binding protein 1) and ELAVL2 [ELAV (embryonic lethal, abnormal vision, *Drosophila*)-like; Hu B]. Analysis of miRNA pairwise correlations revealed the existence of several miRNA co-expression modules that were specific to the training group. These *in vivo* results indicate that significant, dynamic and co-ordinated changes in miRNA expression accompany early stages of learning.

Key words: dicer, learning, microRNA, olfactory discrimination, synaptic plasticity.

INTRODUCTION

Several lines of evidence suggest that miRNAs (microRNAs) regulate aspects of synaptic plasticity by affecting the translation and/or stability of mRNAs that in turn regulate synaptic efficacy, dendritic growth and long-term gene expression (see reviews in Kosik, 2006; Gao, 2008; Christensen and Schratt, 2009). In *Drosophila*, an RISC (RNA-induced silencing complex) component (Armitage) appears to regulate CAMK2a translation and is necessary for long-term olfactory memory (Ashraf et al., 2006). Hippocampal slices exhibiting synaptic potentiation after exposure to tetraethyl ammonium show a large, transient up-regulation of most of the detectable miRNAs assayed at the earliest time examined (15 min), whereas synaptic depression after exposure to DHPG [(S)-3,5-dihydroxyphenylglycine] is associated with a down-regulation of most of the miRNAs at this time point (Park and Tang, 2008).

A large, transient up-regulation of miRNA expression is consistent with findings that dicer and miRNA precursors are highly enriched in association with postsynaptic densities and that synaptic activation [mimicked by NMDA (*N*-methyl-D-aspartate) treatment] results in calpain-mediated activation of dicer RNase III activity and its release from postsynaptic densities, where it may participate in processing the precursors to mature miRNAs (Lugli et al., 2005, 2008;

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Abbreviations: BDNF, brain-derived neurotrophic factor; C_t, threshold cycle value; DHPG, (S)-3,5-dihydroxyphenylglycine; LTP, long-term potentiation; MEF2, myocyte enhancer factor-2; miRNA, microRNA; NMDA, N-*methyl-D-aspartate; pre-miR, miRNA small hairpin precursor; pri-miR, primary miRNA gene transcript; RISC, RNA-induced silencing complex; RT-PCR, reverse transcription-PCR; snoRNA, small nucleolar RNA; TLDA, TaqMan® Low Density Arrays; TOP, terminal oligopyrimidine.*

reviewed in Smalheiser and Lugli, 2009). Theoretically, miRNA/RNA interference pathways have features suggesting that they may regulate long-term changes in gene expression that occur during learning and memory (Smalheiser et al., 2001). However, to date, it is unknown whether changes in expression of miRNAs or other small RNAs occur in the mammalian brain during learning *in vivo*.

In the present study, we employed a hippocampal-dependent olfactory learning task in which adult mice were required to execute a nose poke in a port containing one of two simultaneously present odours in order to obtain a reward. Mice demonstrating discrimination learning were compared with mice that were exposed to the same two odours, but reward was not contingent upon discriminative responding (pseudo-training). In both groups, mice performed the same number of trials, with the same motor responses (nose pokes). Any changes observed in the training versus pseudo-training comparison must specifically reflect the process of learning to associate a specific odour with reward, because other factors such as motor behaviour, odour exposure and novelty were matched. We chose 70% criterion (requiring ~40 min) because that is the earliest point at which mice are behaving significantly different from chance at $P=0.05$. Thus the experimental design examines changes that occur at the onset of the learning process. A preliminary report of these findings was presented at the Society for Neuroscience meeting (Smalheiser et al., 2009).

MATERIALS AND METHODS

Subjects

Subjects were male C57Bl/6J mice (Jackson Laboratory), at 2 months of age. They were housed in groups of four in plastic cages in a climate-controlled animal colony on a normal 14 h light/10 h dark cycle. The mice were maintained on a water deprivation schedule with access to 1.0–2.0 ml of water once a day for at least 5 days before and throughout the training. This schedule reduced body weight by approx. 20% in the first few days, but maintained the mice at a stable weight throughout the study. All testing was done during the light phase. The care and use of animals in this research followed protocols approved by the Institutional Animal Care and Use Committee of the University of Illinois at Chicago and were in accordance with the guidelines of the NIH (National Institutes of Health).

Apparatus

As described previously (Larson and Sieprawska, 2002), the testing chamber was made of black acrylic and consisted of a straight alley 60 cm long and 10 cm wide. The two side (long) walls sloped upward and outward at an angle of 15° off the vertical and were 30 cm high. The end walls were vertical. At each end ('East' and 'West') of the alley were two cylindrical 'sniff ports' (1.5 cm internal diameter) for nose-poke responses (2 cm from the floor and centred 5 cm apart) and a single small cup in the floor for water delivery. The two sniff ports at the west end of the alley were connected to individual air-dilution olfactometers for odour stimulus delivery; all of the sniff ports were equipped for photobeam detection of nose pokes. Water delivery was controlled by electrically driven, Teflon-body solenoid valves (General Valve, Fairfield, NJ, U.S.A.); a microcomputer (PC) detected IR photobeam breaks and activated the valves under custom software control. The whole chamber was enclosed and the ceiling was equipped with an exhaust fan to remove odorants.

The olfactometers were as described previously (Patel and Larson, 2009). Liquid odorants (50 ml) were contained in large glass test tubes (100 ml capacity) fitted with silicone stoppers with two holes drilled to accept clean air input and odorized air output tubing (Teflon, 1/16 inch internal diameter). The odorant tubes were located downstream of flowmeters used to control odorant dilution. The clean air supply (bottled zero air; AGA Gas, Lansing, IL, U.S.A.) in each channel was run at 1.8 litres/min; odorized air was injected into this stream at 0.2 litre/min for an air dilution to 10% of the saturated vapour in the odour tube. Pinch valves (Cole-Parmer, Vernon Hills, IL, U.S.A.) located downstream of the odorant tubes were activated and deactivated to control odorant flow into the odour ports of the testing chamber.

Procedure

All procedures were fully automated and controlled by a computer within each training session.

Nose-poke training

Mice were first trained to execute nose-poke responses for water reinforcement in two 20-trial sessions per day. Each trial was signalled by the extinction of a lamp at the west end of the test box; a nose poke in either west sniff port within 60 s terminated the trial and triggered the delivery of 12.5 µl of water to the water cup. The inter-trial interval was 10 s. Mice were run in two cohorts; nose-poke training continued until all mice in the same cohort reached a criterion of nose pokes on at least 80% of trials in both sessions on at least 3 days. This required 7 days of training for cohort 1 and 5 days for cohort 2. Mice were given a 5-day rest period after the last nose-poke training session before olfactory discrimination training.

Olfactory discrimination

Mice were randomly assigned to three experimental groups: the first group received olfactory discrimination training. Mice were trained in a series of 20-trial sessions in which

each trial began with the simultaneous presentation of two discriminative odours (S+ and S−) to the west sniff ports. The spatial position of the two odours on any given trial was randomly determined except that no more than three identical trials could occur in succession. A nose-poke response at the port carrying the S+ odour (L-carvone) terminated the trial, was scored as correct and was rewarded with a drop of water; a response at the port carrying the S− odour (α-phellandrene) terminated the trial, was scored as incorrect and was not rewarded. Each trial had a maximum duration of 60 s and was followed by a 10 s inter-trial interval. The learning criterion was 14 or more correct trials in a 20-trial session.

The second group received pseudo-training with the same two odours and trial events as in the training group, except that rewards were not contingent upon responding at the correct odour port. A nose-poke response to either odour terminated the trial and was rewarded with a drop of water. Each mouse in the pseudo-training group was yoked to a mouse in the training group in terms of number of training sessions.

The third group simply continued nose-poke training and had no odours presented. Each mouse in this group was yoked to a mouse in the training group in terms of number of training sessions. We will not describe data from this group in any detail in the present paper, since the relevant comparison here is between the learning and pseudo-training groups, but RNA from this group was collected and analysed and will be presented in a separate publication (N. R. Smalheiser, G. Lugli, J. Thimmapuram, E. H. Cook and J. Larson, unpublished data).

Mice were killed immediately after their last training session. Mice were anaesthetized with halothane and decapitated. The brains were rapidly removed and rinsed in PBS. Both hippocampi (including dorsal and ventral regions) were dissected free from each brain, pooled and homogenized in TRIzol® buffer. RNA isolation was carried out using the methods optimized for recovery of tiny RNAs as we have described earlier (Lugli et al., 2008).

A total of nine yoked experiments were carried out. In seven cases, mice achieved the criterion at the end of the third session (taking ~40 min), whereas in the remaining two experiments, the mice achieved the criterion within a single session. Preliminary analysis of miRNA expression indicated that training did cause a striking up-regulation of miRNAs, whether all nine experiments or only the seven typical experiments were included. However, we will present analyses only for the seven yoked experiments where the criterion was reached in three sessions, since they exhibited lower variability in miRNA expression across individuals.

miRNA analysis

High-throughput profiling of 692 miRNAs was carried out using the TaqMan® Rodent MicroRNA Array Set v2.0 (Applied Biosystems, Foster City, CA, U.S.A.). This is a system in which specific primers are used to reverse transcribe mature miRNAs [without recognizing pre-miR (miRNA small hairpin precursor) or pri-miR (primary miRNA gene transcript) precursors], followed by real-time RT–PCR (reverse transcription–PCR) assays carried out in parallel for each miRNA. RT was performed with the TaqMan® MicroRNA Reverse Transcription kit (ABI) and the multiplex RT for TaqMan® MicroRNA Assays (ABI) that consists of two predefined RT primer pools A and B (one for each plate) (ABI) following the manufacturer's protocol. For each RT pool, 1 μg of total RNA was used and the product was diluted 1:75 and mixed 1:1 with TaqMan® Universal PCR Master Mix, No AmpErase® UNG. A 100 μl portion of each mix was dispensed in the appropriate well in the TaqMan® Rodent MicroRNA Array v2.0 [TLDA (TaqMan® Low Density Arrays); ABI] and run to 40 cycles as per the manufacturer's protocol on an ABI 7900HT. Each sample was measured on two plates: A and B; plate A consisted primarily of miRNAs that are annotated as miRNAs in miRBase (http://www.mirbase.org/), whereas plate B consisted primarily of star sequences. The RNA was processed and assayed by an individual who was unaware of group identity; however, an equal number of control samples were processed and assayed on the same day. A sample processed without RT showed no detectable miRNA values.

The primary measurement for a given miRNA is its C_t (threshold cycle value), which indicates the point at which the PCR begins the linear portion (on a logarithmic scale) of its exponential amplification phase. Lower C_t values indicate higher abundance. Note the logarithmic scale for C_t values: a change in mean expression (i.e. ΔC_t) of −1 is equivalent to a 2-fold up-regulation. Primary data cleansing and normalization consisted of the following steps. (i) Determine the threshold for detection. Based on examining samples run on duplicate plates and/or across individuals of the same group to monitor inter-plate reliability, C_t=35 was set as the threshold of detectability. (ii) Normalize values. The miRNA C_t values were normalized to the C_t value of U6 (an endogenous RNA that did not vary significantly across treatment groups and that had low inter-individual variability) by the $\Delta\Delta C_t$ method (adding or subtracting the same value to all miRNAs in a given sample such that the C_t value of U6 equalled its mean value across all samples in all groups). (iii) Remove outliers. We searched for values that are flagged by the machine as unreliable, as well as outliers (defined as measurements that are at least 3 S.D. different from all other measurements of the same miRNA, and occur only once across all samples). No outliers were observed in the present study.

Statistical analysis

Because samples of all three groups were processed and assayed in parallel, statistical analysis was carried out using paired statistics. Although the miRNA expression data appear to be normal in the present study (i.e. passes the Shapiro–Wilk test of normality), we have observed non-normal miRNA expression in previous studies of rat and human brain tissues, and the sensitivity of the normality test may be limited when

there are seven samples per group. Therefore we calculated statistical significance both using the *t* test and the Wilcoxon signed-rank test (two-tailed). The signed-rank test is less sensitive and more conservative than the *t* test, but is appropriate for miRNAs whether or not they follow a normal distribution. The two tests produced highly overlapping lists of significantly affected miRNAs.

Bonferroni correction of statistical significance values is routinely advocated for analyses of mRNA expression profiling. However, the Bonferroni correction assumes that the expression of the vast majority of genes is independent of each other. This situation does not apply in the present study, where many of the miRNAs are closely correlated with each other due to a global up-regulation of miRNA expression as well as the formation of extensive cross-correlation networks (see the Results section). For these reasons, Bonferroni correction is not appropriate for these data. However, additional statistical and biological evidence (see the Results section) strongly supports the conclusion that the set of altered miRNAs reflects a co-ordinated response and does not simply represent a statistical 'tail'.

The TLDA plates included some annotated rat miRNA sequences and we have included these in the data analysis. In some cases, miRBase did not have the corresponding mouse counterpart sequence, even though a homologue is predicted to be encoded in the mouse genome (e.g. rno-mir-664). In other cases, both rat and mouse homologues were included on the TLDA plate, yet the rat homologue had a higher expression in brain than the mouse homologue (e.g. rno-mir-1 and rno-mir-382*). Deep sequencing studies have shown that a significant proportion of miRNA sequences observed within tissues exhibit variants that differ from the canonical miRBase database sequence (e.g. Landgraf et al., 2007), and it is likely in such cases that the rat homologue sequence better reflects the expressed brain miRNA sequences than does the mouse homologue.

RESULTS

Global miRNA changes in the training versus pseudo-training condition

Of 590 RNAs measured on the TLDA A and B plates, 382 were detectably expressed at levels 2-fold or greater above threshold (i.e. the mean C_t value was $\leq$34 in either the training or pseudo-training groups). Most of these were miRNAs, but included on the plates were a few other small RNAs such as Y1 RNA and snoRNAs (small nucleolar RNAs). These were analysed as well. Because the biology of miRNAs might differ between plates A and B, they were analysed separately, although similar trends were observed in both plates.

A global up-regulation of miRNA expression with training was observed on both plates A and B. This effect was apparent both before and after normalizing values using U6 RNA, and can be appreciated in several different ways. (a) Across all expressed miRNAs, the average miRNA expression in the training versus pseudo-training groups was 9.4% higher in plate A and 12% higher in plate B. (b) Comparing individual miRNAs in the training versus pseudo-training groups, 206 of 257 miRNAs on plate A (100 of 125 miRNAs on plate B) were expressed at higher levels in the training group; this was statistically highly significant ($P=1.6\times10^{-17}$). (c) If one considers only miRNAs that changed by a ΔC_t value of 0.5 or greater, 14 miRNAs were up-regulated (compared with two miRNAs down-regulated) on plate A, and this was similarly observed on plate B (eight up-regulated versus one down-regulated). (d) Finally, as shown in Table 1, the number of miRNAs showing significant up-regulation in the training versus pseudo-training condition exceeded the number expected by chance, whereas the number of significantly down-regulated miRNAs was strikingly much less than expected by chance.

At first glance, the global up-regulation of miRNA expression values by 9–12% may appear to be rather small. However, it should be remembered that this is measured across the entire hippocampus. Since learning-specific changes are likely to be restricted to specific subfields or specific cell types within the hippocampus, and may occur within specific dendrites and subcellular compartments (e.g. dendritic spines), the local changes in miRNA expression are likely to be much greater. Although we have no direct evidence that miRNA responses are compartmentalized, within the set of significantly up-regulated miRNAs, the size of the change (ΔC_t) for a given miRNA was strongly and inversely correlated with its baseline expression (C_t value): plate A: $r=-0.76$, $P=0.0011$; plate B: $r=-0.88$, $P=0.0002$ (the pseudo-training group was used as the reference control throughout the present paper). This is consistent with the notion that high-abundance miRNAs tend to be ubiquitously expressed (so only a relatively small fraction of their total miRNA pool in the tissue will be affected with training), whereas miRNAs that are expressed more selectively in neurons and/or in specific locations such as dendritic spines will show the greatest changes.

Table 1 miRNAs showing significant changes in expression in the training versus pseudo-training comparison

An excess number of up-regulated miRNAs was observed relative to the number expected by chance (by *t* test at $P=0.05$ and 0.02; see the list of affected miRNAs in Table 2). Conversely, fewer down-regulated miRNAs were observed relative to the number expected by chance.

P-value	Expected up by chance	Observed up	Expected down by chance	Observed down
Plate A miRNAs, $n=257$				
0.05	6.4	12	6.4	1
0.02	2.6	4	2.6	0
Plate B miRNAs, $n=125$				
0.05	3.1	12	3.1	0
0.02	1.3	7	1.3	0

Another global trend was that the miRNAs showing the largest changes across groups tended to show high inter-individual variability within groups (both in the discrimination learning group and in the pseudo-training group). That is, the mean change in expression of miRNAs (i.e. the ΔC_t value across groups) was correlated with the standard deviation of C_t values across individuals in the training group ($r=-0.39$ for plate A, $r=-0.36$ for plate B) as well as in the pseudo-training control group ($r=-0.30$ for plate A, $r=-0.27$ for plate B). This suggests that the inter-individual variability of miRNA expression values is not simply random noise, but reflects the fact that at least some miRNAs are biologically responsive to a variety of environmental, sensory and/or contextual cues even in the control animals.

Individual miRNA changes in the training versus pseudo-training condition

A total of 30 RNAs that were robustly expressed (i.e. $C_t<34$ in either the training or pseudo-training groups) and that showed significant changes across groups (i.e. $P<0.05$ by either the t test or the signed-rank test) were examined further. This set consisted of 17 miRNAs measured on plate A and 12 miRNAs (plus one significantly affected snoRNA, U87) measured on plate B (Table 2). Except for mmu-mir-297c, which was down-regulated, all were up-regulated with training. The set of significantly altered miRNAs exhibited a wide variety of expression levels and synaptic enrichment ratios; they comprised miRNAs expressed widely in many tissues as well as miRNAs that are primarily expressed in brain and in neurons (results not shown).

A complementary quantile analysis of miRNA expression changes was also carried out. For each treatment group, miRNAs were ordered according to their C_t values and quantile ranks were assigned (i.e. the most abundant miRNA was assigned rank 1, the next most abundant miRNA was rank 2, and so on). Then, for each miRNA, we calculated the difference in quantile ranks between the training and pseudo-training groups. The quantile rank differences followed a normal distribution, suggesting that most miRNAs tended to go up proportionately to the global trend. However, three up-regulated miRNAs showed very large changes in quantile ranks that were significant at $P=0.01$ or better: mmu-mir-10a, rno-mir-1 and 345-3p. The first two also achieved significance using the t test and the signed-rank test (Table 2).

One pair of miRNAs (mir-598-3p and 598-5p) arose from opposite sides of the same hairpin structure, which may

Table 2 miRNAs showing significant changes in training versus pseudo-training comparison

miRNAs	Expression (fold-change over control)	*P*-value		Mean C_t in pseudo-training group
		Signed-rank test	Paired *t* test	
Plate A miRNAs				
mmu-miR-133b	1.841	0.0156	0.0617	31.79
mmu-miR-184	1.3705	0.0156	0.0337	30.38
mmu-miR-324-5p	1.3355	0.0156	0.0178	27.88
mmu-miR-431	1.1128	0.0156	0.0228	26.52
rno-miR-1	1.7392	0.0313	0.0233	30.66
mmu-miR-15a	1.3336	0.0313	0.005	27.61
mmu-miR-19b	1.1638	0.0313	0.0335	23.20
mmu-miR-21	1.2838	0.0313	0.0259	26.36
mmu-miR-29b	1.3256	0.0313	0.0134	26.00
mmu-miR-130b	1.3763	0.0313	0.0339	31.13
mmu-miR-598-3p	1.2078	0.0313	0.0137	28.72
mmu-let-7e	1.1463	0.0469	0.055	23.03
mmu-miR-10a	2.6965	0.0469	0.0252	33.38
mmu-miR-126-3p	1.0691	0.0469	0.053	20.64
mmu-miR-409-5p	1.2382	0.0469	0.0712	30.03
mmu-miR-297c	0.5001	0.0625	0.0288	33.86
mmu-miR-335-5p	1.1399	0.0781	0.0314	27.12
Plate B miRNAs				
mmu-miR-425*	1.4309	0.0156	0.0223	29.85
rno-miR-382*	1.769	0.0156	0.0056	32.55
mmu-miR-592	1.1984	0.0156	0.0273	27.03
U87-4386735	1.1496	0.0313	0.0198	26.19
mmu-miR-154*	1.5137	0.0313	0.0113	31.71
mmu-miR-29c*	1.1841	0.0313	0.0092	29.00
mmu-miR-124*	2.2133	0.0313	0.0195	32.08
mmu-miR-24-2*	1.1347	0.0313	0.0317	27.81
mmu-miR-501-5p	1.8108	0.0313	0.0034	34.70
mmu-miR-485*	1.1589	0.0469	0.0189	27.04
mmu-miR-135a*	1.2879	0.0469	0.0461	29.02
rno-miR-664	1.1201	0.0469	0.0316	25.56
rno-miR-28*	1.7976	0.0625	0.0192	34.96

reflect co-ordinated pri- or pre-miR processing. Three miRNAs (mir-19b, 130b and 721) shared a 5′-seed motif (GUGCA) that has previously been identified as potentially targeting Alu sequences within 3′-UTRs (3′-untranslated regions) (Smalheiser and Torvik, 2006). Three miRNAs (let-7e, mir-196b and rno-mir-196c) shared a different 5′-seed motif (GGUAG). These shared seeds suggest that these miRNAs will exhibit overlap in their corresponding targets (see below).

We sought evidence that the global up-regulation of miRNA expression involved induced transcription of miRNA genes. On the one hand, none of the significantly affected miRNAs (Table 2) were encoded by shared primary miRNA gene transcripts. On the other hand, if one considers the more expanded set of miRNAs that showed relatively large increases with learning (ΔC_t of 0.5 or greater, regardless of *P*-value), we note that ten of the up-regulated miRNAs were encoded at a single chromosomal locus (12F1) that contains a large multi-miRNA gene cluster. Fiore et al. (2009) have shown that 12qF1-encoded miRNAs are stimulated by neuronal activity and mediated by MEF2 (myocyte enhancer factor-2)-dependent transcription. They suggest that all miRNAs in this cluster arise from the same multi-cistronic transcript when driven by MEF2 , and this is consistent with a transcriptional basis of their up-regulation during learning. let-7e and mir-125a-3p appear to arise from the same primary transcript, and several other such cases were observed among up-regulated miRNAs that did not achieve statistical significance. Note that the direction of change was not always consistent; for example, mir-297c, 669a and 467e are all encoded within the same intron but mir-297c showed a down-regulation, whereas the other two were up-regulated. As well, two of the 12qF1 miRNAs showed a down-regulation in contrast to the rest that were up-regulated (results not shown). In summary, the results do suggest that induced transcription contributed to the miRNA response that occurred with training, but the overall response appears to be dominated by induced changes in miRNA processing and turnover.

Target analysis for the training versus pseudo-training conditions

To discern probable biological targets of the affected miRNAs, we combined evidence from 11 miRNA target prediction servers using the miRecords meta-server (Xiao et al., 2009; http://mirecords.umn.edu/miRecords/). For each miRNA that was significantly altered (by either the Wilcoxon signed-rank test or the *t* test) in the training versus pseudo-training comparison, the meta-server was queried to identify targets that are predicted by at least three different prediction servers (to identify highly confident predictions); and for each miRNA queried, the top 30 ranked targets were tabulated (this is a small fraction of the total number of predictions). The meta-server gave predictions for all miRNAs on plate A but only two miRNAs on plate B. Since miRNAs often work in groups to regulate individual targets (e.g. Shalgi et al., 2007), we focused only on targets that were common to more than one significantly affected miRNA listed in Table 2. This approach to creating a target prediction list was automatically generated (and hence unbiased towards any particular type of target) although not comprehensive.

Of 13 targets that were predicted to be hit by two or more significantly affected miRNAs, all appear to be expressed in the hippocampus, and the majority (indicated in boldface) have known roles in synaptic transmission, plasticity or neurogenesis. Three predicted targets were transcription factors: **Zfp238** was predicted to be hit by four different miRNAs and **Sox6** and **Clock**, a gene implicated in neurogenesis (Kimiwada et al., 2008), by two each. Four were proteins with known signalling functions: Wee1 was hit by three miRNAs, and Arhgap12, **Camk2g**, and **Pten**, a protein necessary for long-term depression in the hippocampus (Y Wang et al., 2006), by two each. Two appear to be involved in proteolysis and/or protein turnover, Ankib1 (hit by 3 miRNAs) and Adam12 (2) and two are RNA-binding proteins; **Fmr1** (2) and **Mbnl1** (2). **Bdnf** (brain-derived neurotrophic factor), a growth factor that plays a key role in synaptic plasticity, was predicted to be hit by three miRNAs (rno-mir-1, 10a and 15a). A chloride channel, **Clcn3**, which is regulated by CAMK2a (Huang et al., 2001) and which regulates excitatory synaptic transmission in hippocampal neurons (XQ Wang et al., 2006), was hit by two miRNAs.

Conversely, individual miRNAs were also examined to identify those that hit multiple targets related to synaptic plasticity. The most striking example was also the most highly up-regulated miRNA in the list, r... .0a, whose top 30 target predictions included Bdnf, Camk2b, Creb1 and Elavl2 (Hu B). Another miRNA having five predicted targets related to synaptic plasticity in its top 30 was mir-29b (Camk2g, Eif4e2, Dmnt3a, Pten and Vegfa).

As a supplementary approach to target prediction, we employed a single leading prediction server, TargetScan Mouse (versions 5.0 and 5.1, http://www.targetscan.org/mmu_50/) using default settings to identify targets that contain 8-mer or 7-mer seed complementarity that are conserved across most mammals, but usually not beyond placental mammals (Friedman et al., 2009). (Note that TargetScan did not give predictions for mir-598-3p, nor for miRNAs on the B plate except for mir-501-5p and 592.) Among the predicted targets listed for each miRNA, we noted targets that were common to multiple miRNAs or that had high intrinsic interest, and then entered queries for each putative target separately, counting only the predicted miRNA interactions. This approach examined many more predicted targets per miRNA than the miRecords analysis (above), but was not unbiased because we focused on particular targets of interest. Again, prominent among the predicted targets were transcription factors: **Bach2** (hit by 5 different affected miRNAs); HoxA3, E2F7 and **Fosl2** (fra-2) (4 each); **Camta1** that is correlated with human memory performance (Huentelman et al., 2007) (4); **Mecp2** (4); and Esrrg and **Creb1** (2 each). Proteins with known signalling

functions included **Pafah1b1**, **Pten** and Spry4 hit by five miRNAs each, **Calm1** (calmodulin) (3), **Bcl2l2** (Bcl-W) (3), **Robo2** (3), **Dlg2** (3), Nufip2, an FMRP (fragile X mental retardation protein)-interacting protein (3), **Camk2g** (2), **Cask** (2), **Cpeb1** (2), **Dlgap2** (2) and **Dnmt3a** (2). RNA-binding proteins included Qk (Quaking), a glial-specific protein hit by six miRNAs; followed by **Dcr1** (dicer-1) the enzyme that processes pre-miRs to mature miRNAs (3); **Elavl1** (Hu R), which is known to associate with RISC (Bhattacharyya et al, 2006) (3); **Fmr1** (2); Eif2c4 (Argonaute homologue isoform 4) a core component of the RISC complex (2); **Elavl2** (Hu B) (2) and **Elavl4** (HuD) (2). An RNA-permeable pore protein expressed in the brain, Sidt2, was also hit by two miRNAs. A microtubule-stabilizing protein, **Mtap4**, was hit by three miRNAs. Finally, the list of putative targets included several receptors including **Grm7** (metabotropic glutamate receptor 7) (2) and Calcr (2), as well as several growth factors including **Bdnf** (3) and **Vegfa** (3).

Pairwise correlation analysis of miRNAs co-expressed across individuals specifically in the training group

In contrast with identifying miRNAs that show significant mean differences in expression across treatment groups, pairwise correlation analysis (as done here) identifies pairs of miRNAs whose expression varies up or down in parallel across individuals of the same treatment group. Pairs of miRNAs may be co-expressed because they are encoded by the same pri-miR or pre-miR precursor or because they are driven by the same transcription factor or regulated by the same cellular mechanism(s). In the present study, we are interested in identifying pairs of miRNAs that are significantly correlated in the training group, but not significantly correlated in the control groups. This provides a novel means of detecting co-ordinated changes in miRNA expression that are specifically associated with learning.

In this analysis, the list of plate A miRNAs was first filtered to include only those miRNAs that had robust expression (i.e. whose C_t value was <35 in all individuals in all treatment groups, including the nose-poke control group). The C_t values for each miRNA were normalized to the global mean C_t value of the same individual (averaged over all miRNAs), in order to remove correlations due to inter-individual differences in overall miRNA content. Next, all miRNAs were examined pairwise and the Pearson correlation coefficient was computed for each of the treatment groups separately. For seven animals per treatment group, only correlations of $r=0.755$ or greater were significantly different from 0 (at $P=0.05$). Finally, we identified pairs of miRNAs that satisfied the following criteria. (i) They exhibited a significant positive correlation in the training group. (ii) The correlation coefficients in the pseudo-training and nose-poke groups were much lower than that in the training group (r-training – r-pseudo and r-training – r-nose >0.7). (iii) The pairs did not exhibit a significant negative correlation in the control groups (r-pseudo and r-nose >-0.755).

A total of 130 pairs of miRNAs satisfied these criteria and were displayed as a visualization network, in which individual miRNAs are nodes and are linked whenever the two miRNAs show a significant correlation in the training group. This included not only many of the miRNAs that were significantly up-regulated during training, but many others that did not show significant mean differences as well. Of particular interest (and high confidence) is the subset consisting of miRNAs that are each positively correlated with at least two other miRNAs within the network, forming three distinct clusters (Figure 1). The central cluster has a particularly densely interconnected core, having two hubs (mir-188-5p and 489) surrounded by 15 miRNAs that are correlated with both hubs. Several of the miRNAs in the central cluster were significantly up-regulated by training (mir-15a, 19b, 21 and 335-5p), whereas others were up-regulated but did not achieve significance (mir-665), and others (notably the two hubs mir-188-5p and 489) did not show any mean difference in the training versus pseudo-training comparison (see Supplementary material at http://www.asnneuro.org/an/002/an002e028.add.htm).

These findings are noteworthy because they demonstrate that miRNAs did not merely go up and down individually in their expression, but comprised networks that underwent a co-ordinated reorganization during training. The core of the central cluster, in particular, appears to comprise a co-expressed module. This phenomenon may be explained if the miRNAs in the module are driven by transcription factor(s) that are induced specifically during the onset of learning. However, relatively little is known at present concerning which transcription factors drive miRNA genes, so it is premature to infer which factor(s) may be responsible. Alternatively, a variety of proteins are known to regulate defined subsets of miRNA genes (e.g. Trabucchi et al., 2009); hence, training-related changes in one or more of such proteins might also affect miRNA pairwise correlations. Co-expressed miRNAs may be expected to hit related or overlapping sets of targets (Zhou et al., 2007).

DISCUSSION

The present study demonstrates that rapid, dynamic and co-ordinated changes in miRNA expression occur in the hippocampus during a hippocampal-dependent learning paradigm *in vivo*. The two-odour olfactory discrimination task employed a pseudo-training control group that was designed to isolate effects specifically due to learning from changes that may possibly accompany novelty, odour pair exposure or motor behaviour. We examined an early phase in the learning process (70% correct responses in a 20-trial session) in order to measure miRNA changes that accompany

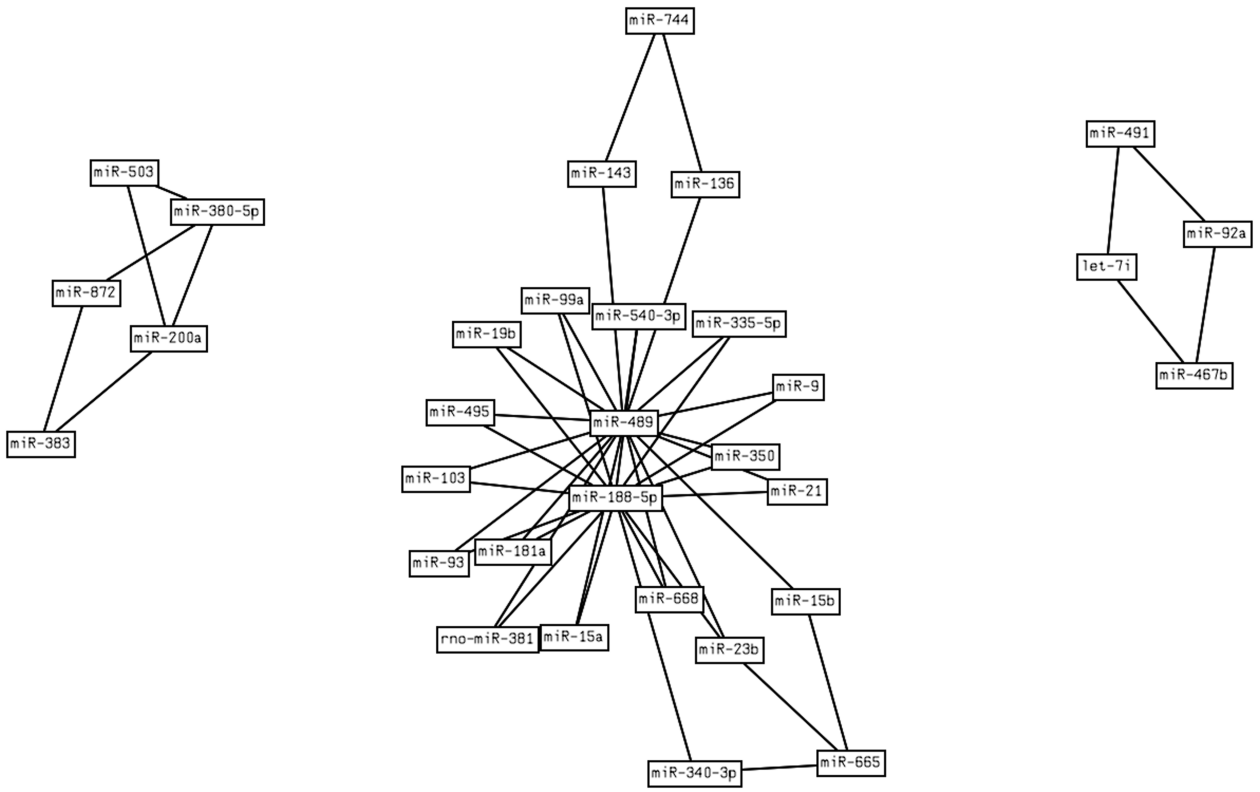

Figure 1 miRNAs that are positively and significantly correlated with at least two other miRNAs within the training group, but are not correlated with each other in the control groups
See the Results section for details. Three distinct modules are formed; the central module has a particularly densely interconnected core, having two hubs (mir-188-5p and 489) surrounded by 15 miRNAs that are correlated with both hubs.

the onset of learning. The present study provides the first evidence that miRNA expression is specifically altered during an *in vivo* learning paradigm in mammals.

A global up-regulation of miRNA expression was observed with training, of which 28 miRNAs showed significant up-regulation at $P=0.05$ or better (and one was down-regulated). Although the mechanism is unknown, this observation is consistent with previous results suggesting that processing of miRNA precursors to mature miRNAs can be rapidly induced by synaptic activity. NMDA-mediated stimulation of hippocampal slices or calcium-mediated stimulation of synaptoneurosomes can lead to rapid calpain-dependent cleavage of dicer that activates its RNase III activity and that may be expected to lead to a burst of processing of miRNA precursors to mature miRNAs (Lugli et al., 2005, 2008). Park and Tang (2008) demonstrated that 'chemical LTP (long-term potentiation)' elicited in hippocampal slices leads to a global increase in miRNA levels at 15 min (the earliest time examined). They also noted that DHPG stimulation (associated with long-term depression) led to a rapid initial decrease in miRNA levels, emphasizing that stimuli may also induce rapid turnover of miRNAs. Rapid activity-dependent transcription of miRNA genes can also occur (Fiore et al., 2009; Nudelman et al., 2009) and may have contributed to the up-regulation as well.

Besides identifying miRNAs that showed significant mean differences in expression across treatment groups, we also carried out pairwise correlation analysis to identify pairs of miRNAs whose expression varied up or down in parallel across individuals belonging to the same treatment group. Several modules of co-expressed miRNAs were detected that were highly correlated in the training group, but not at all correlated in the control groups. This independent statistical method strengthened our conclusion that miRNAs were affected during training, not simply as individual entities, but in a co-ordinated manner. As well, pairwise correlation analysis revealed that factors controlling miRNA expression were reorganized during learning, even for some miRNAs that did not alter their mean expression levels.

The effects of miRNAs on gene regulatory networks are not straightforward. miRNAs bind mRNAs, repressing their translation and/or sequestering them in P bodies (Bushati and Cohen, 2007), but recent studies show that miRNAs may enhance translation under some conditions (Vasudevan et al., 2007), and may repress or stimulate transcription of target genes (Kim et al., 2008). Transcription of miRNA genes may potentially affect the transcription of nearby mRNAs as well (Smalheiser, 2003). Moreover, miRNAs work in concert with other miRNAs and with transcription factors to form extensive feedback and feedforward circuits (Zhou et al.,

2007). Especially in the brain, many miRNAs are co-regulated positively (rather than inversely) with their targets (Tsang et al., 2007; Liu and Kohane, 2009). Relatively few miRNA targets have been biologically validated to date, generally under non-physiological conditions or in non-neural systems. Different miRNA target prediction algorithms give hundreds to thousands of predicted targets per miRNA, yet often show little agreement with each other (e.g. Bentwich, 2005).

For all of these reasons, predicting the net effects of a given change in miRNA expression, and predicting which targets may be regulated by a given set of miRNAs, must be considered as provisional exercises. Of the 13 targets that were predicted by the miRecords meta-server to be hit by two or more significantly affected miRNAs, all appear to be expressed in the hippocampus, and nine have known roles in synaptic transmission, plasticity or neurogenesis. This is a striking effect and is not simply due to bias in selecting candidate targets. The TargetScanMouse server also identified many other conserved targets that are predicted to be hit by two or more miRNAs significantly affected by training. Some of these may be antagonistic to synaptic remodelling, and conceivably might need to be down-regulated at the onset of the learning process; for example, Quaking, a glial protein involved in myelination, Mtap4, a microtubule-stabilizing protein, or Pten, a phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase that is involved in long-term depression (Y Wang et al., 2006). Others have well-known positive roles (e.g. fragile X mental retardation protein, Fmr1). Predicted targets also included proteins that are part of the miRNA processing pathway (e.g. dicer itself, Dcr1). Perhaps the single most interesting miRNA affected by training was mir-10a, which exhibited the largest fold-change of expression as well as the largest change in quantile ranks. mir-10a is predicted to hit numerous plasticity-related targets including Bdnf, Camk2b, Creb1 and Elavl2 (Hu B). Ørom et al. (2008) have reported that mir-10a has a positive effect on general protein translation by binding to the 5′-UTR of ribosomal 5′ TOP (terminal oligopyrimidine) mRNAs and enhancing their translation. Since up-regulation of 5′ TOP mRNA translation occurs during LTP within neurons (Gobert et al., 2008), up-regulation of mir-10a could potentially contribute to an overall activity-dependent burst of protein translation that occurs e.g. within dendritic spines.

Further studies are needed to learn exactly how miRNA responses fit into the overall dynamic scheme of gene and protein changes that occur during learning. For example, although Bdnf mRNA is induced in the hippocampus during learning (Hall et al., 2000) and release of Bdnf protein is induced after LTP elicited by perforant path stimulation in the rat dentate gyrus (Gooney and Lynch, 2001), the tissue levels of Bdnf protein are unaffected by such stimuli (Walton et al., 1999). Bdnf mRNA and protein levels show little correlation in human prefrontal cortex, indicating that Bdnf is subject to separate transcriptional and translational regulation by miRNAs and other factors (Mellios et al., 2008a, 2008b). Targets such as fragile X mental retardation protein undergo a complex cycle in which an initial burst of translation is followed by a down-regulation (Gabel et al., 2004).

In conclusion, the overall picture that emerges from our study is that at least some miRNAs in the brain are dynamically sensitive to environmental, sensory and contextual cues. This opens the door to more detailed analyses of how up-regulation of a set of miRNAs affects gene expression pathways during learning. Further mechanistic studies are needed to localize changes within individual hippocampal regions, cell types and subcellular compartments, and to characterize the temporal profile of miRNA changes at various stages in learning and memory.

FUNDING

This work was supported by grants from NIH [R21MH081099, R0168777 and R01DC005793] and the Stanley Medical Research Institute.

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Received 5 December 2009/14 January 2010; accepted 18 January 2010

Published as Immediate Publication 18 January 2010, doi 10.1042/AN20090055